

# Effect of AT-125 on in situ renal $\gamma$ -glutamyltransferase activity

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## 1. INTRODUCTION

$\gamma$ -Glutamyltransferase ( $\gamma$ -GT) is a membrane-bound glycoprotein [1,2] reacting with substrates present in the extracellular milieu [3]. Renal  $\gamma$ -GT is present on the brush border of both convoluted and straight portions of the proximal tubule [3,4] although some 2.5-times more concentrated on the latter [4,5]. In addition a small but significant portion of the total renal activity is present in the antiluminal region [6,7] associated at least in part, with the microvascular compartment [8]. The fact that the bulk of the total renal  $\gamma$ -GT is present on the luminal border and that the substrate delivery to this site is limited by the glomerular filtration rate suggested that the reaction catalyzed by  $\gamma$ -GT at this site would be substrate limited; in contrast, the high substrate delivery and low enzyme level suggested that the reaction might be enzyme-limited at the antiluminal site. To examine this possibility we employed AT-125 (L-( $\alpha$ -amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid) as an inhibitor of the in situ  $\gamma$ -GT and measured the response at both luminal and antiluminal sites as a function of total enzyme activity.  $\gamma$ -Glutamyl-*p*-nitroanilide ( $\gamma$ -GpNA) rather than glutathione was used as the substrate to avoid the complication introduced by the glutathione oxidation [9]. The response was evaluated in terms of *p*-nitroaniline (*p*-NA) formation (largely a function of the antiluminal site) and in terms of filtered  $\gamma$ -GpNA escaping into the urine (a function of the brush border enzyme activity). The results to follow are consistent with the interpretation that the blood side reaction is enzyme-limited, and therefore most susceptible to inhibition, and that the brush border enzyme is substrate-limited, requiring

nearly complete inhibition for a response to be observable.

## 2. MATERIALS AND METHODS

Kidneys were obtained from male Sprague-Dawley rats (350–450 g body wt). The kidneys were isolated as in [10] and perfused with an artificial plasma solution [11] containing [ $^{14}$ C]methoxyinulin and 2 mM  $\gamma$ -GpNA at pH 7.4 (95% O<sub>2</sub>:5% CO<sub>2</sub>). Perfusate and urine samples were collected at timed intervals and analyzed for  $\gamma$ -GpNA and *p*-NA concentration [6] and  $^{14}$ C by liquid scintillation spectrometry; glomerular filtration rate (GFR) was estimated from the urine: plasma  $^{14}$ C ratio  $\times$  urine flow. Left non perfused and right perfused kidneys from each animal were homogenized and assayed for  $\gamma$ -GT activity [12]; homogenate protein concentration was analyzed as in [13]. Total renal  $\gamma$ -GT activity determined in vitro is taken as spec. act.  $\times$  renal protein content; in situ activity of the perfused kidney is taken as the amount of *p*-NA formed (perfusate + urine) unit time per kidney. For inhibition studies, kidneys were pre-perfused for 30 min with AT-125 (kindly supplied by Dr Ruth Davis, National Institute of Health) at 1.8–24 nmol/kidney after which the perfusate was replaced by means of a 3-way stop-cock [14] with a fresh media containing  $\gamma$ -GpNA. Renal tissue levels of  $\gamma$ -GpNA were measured after perfusion of control and AT-125-treated kidneys; kidneys were promptly homogenized at the termination of perfusion in 10% ice-cold trichloroacetic acid, neutralized and analyzed enzymatically using purified  $\gamma$ -GT [6];  $\gamma$ -GpNA content is expressed as  $\mu$ mol/g wet wt after subtracting the pre-formed *p*-NA content.

## 3. RESULTS

Fig. 1 shows the fall in perfusate  $\gamma$ -GpNA and rise in  $p$ -NA over the perfusion course in both the perfusate and urine compartments. The control rate of  $p$ -NA formation over this time course was  $1.53 \pm 0.26 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ , representing  $p$ -NA released into the perfusate + urine; after AT-125, the rate fell to  $< 10\%$ ,  $0.12 \pm 0.05 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$ . The rate of  $\gamma$ -GpNA disappearance from the perfusate in control,  $1.75 \pm 0.15 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  was slightly greater than  $p$ -NA formation due to the synthesis of  $\gamma$ -glutamylpeptides appearing in the perfusate [6]; the disappearance in the AT-125-treated kidneys,  $0.60 \pm 0.36 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  cannot be attributed to tissue accumulation (tissue levels  $1.77 \pm 0.15$  and  $1.80 \pm 0.21 \mu\text{mol/g}$  in control and AT-125-treated kidneys). Rather this decrease reflects the loss of  $\gamma$ -GpNA into the urine as well as the small residual activity of  $\gamma$ -GT in converting  $\gamma$ -GpNA to  $p$ -NA. Renal levels of  $\gamma$ -GT were  $1064 \pm 91 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$  in controls and  $11 \pm 3 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$  after perfusion in kidneys exposed to 6 nmol AT-125.

The renal handling of filtered  $\gamma$ -GpNA is shown in table 1. In the control kidneys  $\gamma$ -GpNA was delivered to the brush border enzyme at the rate of  $0.69 \pm 0.25 \mu\text{mol/min}$  which is  $< 50\%$  the rate of

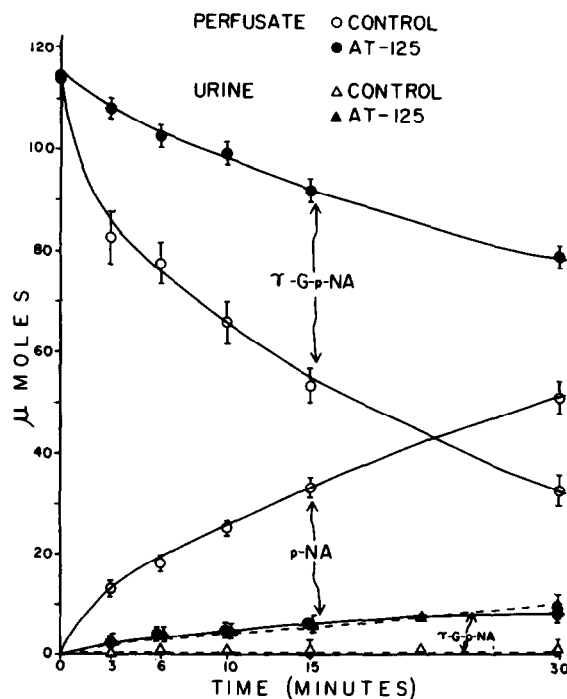


Fig.1. Effect of 6 nmol AT-125 on  $\gamma$ -GpNA disappearance from the perfusate and appearance in the urine as well as on the formation of  $p$ -NA. Results are means  $\pm$  SEM from 4 control and 4 AT-125-treated perfused kidneys.

Table 1  
Influence of AT-125<sup>a</sup> on the excretion of  $\gamma$ -GpNA

	$\gamma$ -GpNA <sub>p</sub> ( $\mu\text{mol/ml}$ )	GFR (ml/min)	Filtered ( $\mu\text{mol/min}$ )	$\dot{Q}_\mu$ (ml/min)	$\gamma$ -GpNA <sub><math>\mu</math></sub> ( $\mu\text{mol/ml}$ )	Excreted ( $\mu\text{mol/min}$ )	FE <sup>b</sup> $\gamma$ GpNA (%)	$\gamma$ GpNA Urine Perfusate
Control (4)								
$\pm$	0.810	0.550	0.690	0.290	0.110	0.020	0.730	0.070
SEM	0.174	0.080	0.250	0.044	0.065	0.008	0.730	0.049
AT-125 (4)								
$\pm$	1.400	0.400	0.620	0.250	1.640	0.340	61.670	1.250
SEM	0.250	0.060	0.110	0.063	0.345	0.060	6.229	0.395
P	$< 0.025$	n.s.	n.s.	n.s.	$< 0.005$	$< 0.0005$	$< 0.0005$	$< 0.01$

<sup>a</sup> Kidneys perfused with 6 nmol AT-125;(4) equals number of kidneys

<sup>b</sup> Fractional excretion of  $\gamma$ -GpNA = Excreted  $\gamma$ -GpNA/Filtered  $\gamma$ GpNA  $\times 100$

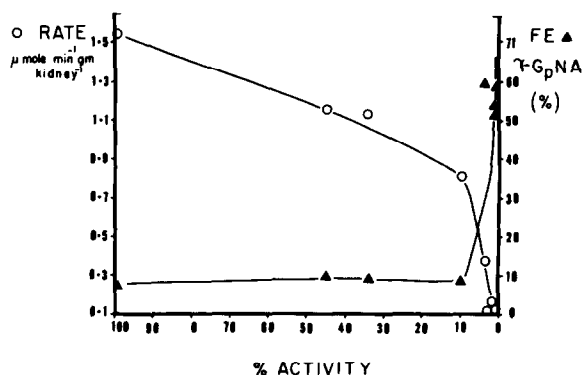


Fig.2. Relationship between total renal  $\gamma$ -GT activity and  $p$ -NA formation (○) and  $\gamma$ -GpNA excretion (▲); FE = fraction of filtered  $\gamma$ -GpNA excreted. Renal  $\gamma$ -GT activity was reduced by pre-perfusion with increasing amounts of AT-125 (see text for details).

$p$ -NA formation,  $1.52 \mu\text{mol/min}$ . Of the  $\gamma$ GpNA delivered, only 0.73% was excreted while the urine to perfusate  $\gamma$ -GpNA ratio, U/P, was  $0.07 \pm 0.05$  indicative of a steep gradient between perfusate and urine. With AT-125, 62% of the filtered  $\gamma$ -GpNA was excreted with a U/P ratio  $> 1$  indicating that  $\gamma$ -GT activity is at least in part responsible for this gradient; renal tissue  $\gamma$ -GpNA to urine  $\gamma$ -GpNA concentration ratios fell from  $21 \pm 13$  in control to  $2.1 \pm 1.1$  with AT-125.

The affect of exposing the kidney to various amounts of AT-125 on total renal  $\gamma$ -GT activity,  $p$ -NA formation and  $\gamma$ -GpNA excretion is shown in fig.2. After exposure to 1.8 nmol AT-125 total renal activity fell to 45% while  $p$ -NA formation dropped to  $1.17 \mu\text{mol/min}$  and  $\gamma$ -GpNA excretion remained unchanged. With 4 nmol AT-125, total  $\gamma$ -GT activity dropped 90% with a further reduction in  $p$ -NA formation and little increase in  $\gamma$ -GpNA excretion. However, at greater AT-125 levels, renal  $\gamma$ -GT activity was virtually eliminated resulting in a large rise in  $\gamma$ -GpNA excretion, from 7% to  $> 50\%$  of that filtered. At these levels of inhibition the urine to plasma  $\gamma$ -GpNA ratio was  $> 1$ , similar to results in table 1.

#### 4. DISCUSSION

$\gamma$ -Glutamyltransferase is present in the rat kidney in great excess. Some appreciation of this excess can

be gained from considering  $\gamma$ -GpNA conversion under optimum conditions, i.e., kidney homogenate, in which substrate would not be rate limiting. Under such conditions the total capacity to utilize  $\gamma$ -GpNA approaches  $200 \mu\text{mol/min kidney}$  (spec. act.  $1000 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1} \times \text{kidney protein } 200 \text{ mg}/1.5 \text{ g kidney}$ ). In contrast the isolated kidney perfused with similar  $\gamma$ -GpNA levels utilized only  $1.5 \mu\text{mol/min}$  indicating the in situ enzyme is clearly limited by the conditions imposed by the intact preparation. It was to gain a clearer understanding of the relationship between renal enzymology and function which lead to this study.

The delivery of substrate to the kidney is limited by the perfusate flow (here  $30 \text{ ml/min}$ )  $\times$  perfusate [ $\gamma$ -GpNA] which was initially  $2 \text{ mM}$ . However, delivery of substrate to the brush border enzyme is further constricted by the  $GFR$  ( $\sim 0.5 \text{ ml/min}$ )  $\times$  perfusate [ $\gamma$ -GpNA]. Thus, under these conditions, the brush border site, containing some 80% [6] of the total kidney enzyme receives  $< 2\%$  of the  $\gamma$ -GpNA delivered to the kidney; in contrast the antiluminal site receives 98% of the delivered substrate. Consequently the reaction occurring at the brush border and antiluminal site might be expected to be substrate- and enzyme-limited, respectively. An inhibitor that had access to both sites and that binds (stoichiometrically) to the  $\gamma$ -glutamyl donor site [15] might be expected to reveal this asymmetrical distribution. Giving AT-125 at a dose calculated to inhibit  $\sim 50\%$  of the total renal enzyme significantly reduced  $p$ -NA formation, occurring predominantly at the enzyme-limited antiluminal region, and had no effect at the brush border where the reaction is substrate-limited (fig.2). Completely inhibiting  $\gamma$ -GT with 6 nmol AT-125 results in virtual elimination of  $p$ -NA formation and maximal  $\gamma$ -GpNA excretion; although 40–50% of the filtered  $\gamma$ -GpNA is not excreted and therefore reabsorbed, this appears to be a passive process since the U/P ratio  $> 1$ . Furthermore, the elimination of perfusate to urine  $\gamma$ -GpNA concentration differences indicates that  $\gamma$ -GT activity maintains this gradient suggesting that this enzyme functions in vivo to ensure similar gradients of the natural substrates.

In vivo the delivery of extrarenal substrate is limited by the renal plasma flow and glomerular filtration rate. Under these conditions some 80% of the delivered glutathione is removed [16,17] while inhibiting  $\gamma$ -GT 90% lowers this to 30% as a conse-

quence of excretion [17] similar to fig.1. Thus the extraction fraction, 80%, exceeds the filtration fraction, 20–30% in vivo, an excess which is eliminated by inhibiting  $\gamma$ -GT. These observations are consistent with antiluminal  $\gamma$ -GT activity being responsible for 50% of the removed glutathione. Secretion of glutathione from blood to urine might also account for the high extraction fraction; however, inhibition of  $\gamma$ -GT by AT-125 should diminish the extraction if it was in fact dependent upon secretion. Furthermore, in the isolated perfused kidney the excretion of the  $\gamma$ -glutamyl donor never exceeds its filtration rate after  $\gamma$ -GT inhibition indicating that at least for this substrate secretion is not apparent. Of course, it does not exclude the possibility that AT-125 affects both  $\gamma$ -GT and a secretory system for  $\gamma$ -glutamyl substrates. Nevertheless, this study points to a significant role of the antiluminal site in utilization of  $\gamma$ -glutamyl substrates and that this site, in view of the limited enzyme available relative to substrate, is most susceptible to inhibition. In contrast, the luminal enzyme is present in  $\geq 10$ -fold excess requiring near complete inhibition to reveal a maximal response.

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